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Glycosylated Nanoparticles as Efficient Antimicrobial Delivery Agents

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Synthetic polymer nanoparticles that can be tailored through multivalent ligand display on the surface, while at the same time allowing encapsulation of desired bioactive molecules, are especially useful in providing a versatile and robust platform in the design of specific delivery vehicles for various purposes. Glycosylated nanoparticles (glyco-NPs) of a poly(n-butyl acrylate) (pBA) core and poly(N-2-(β -D-Glucosyloxy)ethyl acrylamide) (p(N β GlcEAM)) or poly(N-2-(β -D-galactosyloxy)ethyl acrylamide) (p(N β GalEAM)) corona were prepared *via* nanoprecipitation in aqueous solutions of preformed amphiphilic glycopolymers. Well-defined block copolymers

of (poly(pentafluorophenyl acrylate) (pPFPA) and pBA were first prepared by RAFT polymerization followed by post-polymerization functionalization with aminoethyl glycosides to yield p(N β GlcEAM-*b*-BA) and p(N β GalEAM-*b*-BA) which were then used to form glyco-NPs (glucosylated and galactosylated NPs, Glc-NPs and Gal-NPs, respectively). The glyco-NPs were characterized by dynamic light scattering (DLS) and TEM. Encapsulation and release of ampicillin, leading to nanoparticles that we have termed ‘glyconanobiotics’, were studied. The ampicillin-loaded glyco-NPs were found to induce aggregation of *Staphylococcus aureus* and *Escherichia coli* and resulted in antibacterial activity approaching that of ampicillin itself. This glyconanobiotics strategy represents a potential new approach for the delivery of antibiotics close to the surface of bacteria by promoting bacterial aggregation. Defined release in the proximity of the bacterial envelope may thus enhance antibacterial efficiency and potentially reduce the quantities of agent required for potency.

Over the last century, morbidity and mortality as a consequence of infectious diseases have decreased drastically, due to the wide-spread development and deployment of vaccines and antimicrobial agents.¹ However, bacterial resistance to our available antibiotic repertoire is now reaching a critical level, potentially bringing about a post-antibiotic era.² Considerable effort has been expended to overcome this problem through the discovery of new antibiotics and chemical modification of existing antibacterial drugs. Nevertheless, the development of new antimicrobial drugs is unlikely to outpace the emergence of microbial pathogen resistance. The continued evolution of antimicrobial resistance mechanisms has prompted the scientific community to seek longer-term solutions to this ever-growing problem.³ Metallic nanomaterials (e.g. Ag, Au, and Cu) have been found to display strong antimicrobial properties.⁴ Nevertheless, their applications as antimicrobial agents are limited by their potential toxicity to human cells as a result of their unusual physicochemical properties and/or physical or chemical production techniques.⁵ Encapsulation or conjugation of antibiotics into different classes of nanocarriers has been recently represented as a potential approach to combat infectious diseases.⁶⁻⁸ This approach can not only offer efficient intracellular delivery to pathogens but also help in controlling the amount and frequency of drug dosage and hence reduces the toxicity associated to therapy and may overcome bacterial resistance.

Strategies that do not kill the pathogens yet interfere with their pathogenic mechanisms may provide a promising alternative. One such strategy is anti-adhesion therapy, which interferes with the early stages of infection in which pathogens attach to the mammalian cell surface.⁹⁻¹¹ This adhesion is often mediated by protein-carbohydrate interactions: proteins on the pathogen binding to displayed carbohydrate structures or receptors on the eukaryotic cell. These interactions are often highly specific and determine the preference of the pathogen for certain

tissue types, known as tropism. Free carbohydrates can be harnessed to interfere with bacterial attachment, thus preventing initial colonization and subsequent infection. This principle is operative daily as part of the innate immune system, for example human breast milk contains many oligosaccharides that act as anti-adhesives.¹² However, the limited affinity of monovalent carbohydrates for target proteins that are often multivalent hinders the application of this approach. Therefore, designing multivalent glycosylated constructs that can inhibit protein-carbohydrate interactions may be productive in finding a way forward.

During the last decade, nanoparticles presenting carbohydrate/glycan functionalities at their surfaces have been reported to exhibit antibacterial actions towards different classes of bacteria including Gram-positive, Gram-negative and mycobacteria [ref]. Most of these reports focused on using nanoparticles with an inorganic core, such as gold, silver, iron oxide, silica, copper, bismuth, palladium, and platinum.¹³ A major reason for the special interest in these inorganic materials is their attractive physical properties, such as plasmonic effects, luminescence and/or magnetic susceptibility which make them especially useful for both imaging and therapeutic (theranostic) applications. One of the most studied examples of carbohydrate-mediated targeting that leads to bacterial growth inhibition exerted by aggregation has been shown with mannosylated silver and gold nanoparticles, interacting with fimbriated *E. coli* strains.^{14, 15} Bacterial growth inhibition has also been demonstrated with non-targeting glycosylated nanoparticles, where the carbohydrate moieties serve as stabilising and/or solubilizing agents. For example, silver nanoparticles functionalized with kocuran (an exopolysaccharide produced by *Kocuria rosea* strain BS-1) were probed against a range of bacterial strains, of which *S. aureus* was affected the most.¹⁶ The binding affinity and specificity of glycosylated nanoparticles to many bacterial strains is not well-studied in the literature and needs further attention.

Here, we report the construction of polymeric glycosylated nanoparticles (glyco-NPs) that encapsulate an antibiotic (ampicillin) as an amalgamated system, which we refer to as *glyconanobiotics*. Our hypothesis is that antibiotic delivery will be enhanced if the nanoparticle delivery vehicle is capable of binding to, and aggregating, the bacteria, and therefore releasing the antibiotic in the proximity of the bacterial surface. As an example, the pili of *E. coli* contain at their tips the FimH protein which has binding specificity for glucose and mannose.¹⁷ Consequently, multivalent glucosylated nanoparticles loaded with antibacterial agent could form such a glyconanobiotic delivery system. Ampicillin was used in this study as a well-characterized and potent antibiotic. It is a semi-synthetic derivative of penicillin, with a relatively short-term stability in aqueous solutions.¹⁸ It is used clinically for the treatment of a broad range of bacterial infections.^{19, 20} Improvement of its activity and reduction of its allergic and toxic reactions have been achieved by means of topical formulations²¹ and the use of liposomal nanoparticles as passive delivery systems.^{22, 23} Moreover, from the pharmaceutical application perspective, these liposomal nano-carriers provide endocytosable formulations for intracellular chemotherapy, since β -lactam antibiotics do not diffuse readily through the lysosomal membrane because of their ionic character at neutral extracellular or cytoplasmic pH.²⁴ Polymeric nanoparticles offer, over liposomes, certain advantageous features with respect to chemical and biological stability and prolonged circulation times in the bloodstream.²⁵ Furthermore, they can be prepared in such a way that they present on their external surface biologically active functionalities,^{26, 27} for example carbohydrates as employed in this study.

EXPERIMENTAL METHODS

Typical synthesis of amphiphilic glycopolymers

PFPA (476 mg, 2 mmol), benzyl 2-hydroxyethyl carbonotrithioate (32 mg, 0.13 mmol), AIBN (4.5 mg, 0.025 mmol) and benzene (5 mL) were placed into a Schlenk flask equipped with a stirrer bar. After degassing by purging with nitrogen under an ice bath for 30 min, the solution was heated to 70 °C and stirred for 6 h. Conversion of monomer to polymer was determined by ^1H NMR spectroscopy. The polymerization reaction was quenched by cooling and exposure to air. n-Butyl acrylate (2.76 g, 21.6 mmol) and AIBN (4.5 mg, 0.025 mmol) in benzene (8 mL) were added to the crude PFPA homopolymer solution. After degassing by purging with nitrogen under an ice bath for 30 min, the mixture was heated to 70 °C and stirred for 18 h. After quenching the reaction, the solvent was removed under reduced pressure and the product was reprecipitated twice from THF into cold methanol (at 0 °C). The block copolymer was dried under reduced pressure to yield a yellow powder which was fully characterized by SEC, ^1H and ^{19}F NMR spectroscopy, and ATR-FTIR spectroscopy.

The obtained PFPA/BA block copolymer was first dissolved in toluene (30 mL) in the presence of a large excess of AIBN (30 eq.). After degassing by purging with nitrogen under an ice bath for 30 min, the mixture was heated to 70 °C and stirred for 6 h. After quenching the reaction, the solvent was removed under reduced pressure and the product was reprecipitated from THF into cold methanol (at 0 °C) to yield p(PFPA₁₅-*b*-BA₁₂₀) as an off-white powder. 2'-aminoethyl- β -D-glucopyranoside (100 mg, 0.45 mmol) was mixed with triethylamine (100 μL) in water (1 mL). While stirring, the sugar solution was added slowly to p(PFPA₁₅-*b*-BA₁₂₀) (200 mg) solution in DMF (3 mL). The mixture was stirred at 30 °C for 18 h. The product was then dialyzed against deionized water for 24 h and freeze-dried to yield p(N β GlcEAM₁₅-*b*-BA₁₂₀) which was fully characterized by SEC, ^1H and ^{19}F NMR spectroscopy, and ATR-FTIR spectroscopy.

In vitro drug release from ampicillin-loaded glyco-NPs

Ampicillin-encapsulated glyco-NPs were prepared using the nanoprecipitation method with the addition of 0.5 mg of ampicillin to the organic phase. Samples with a specific amount of ampicillin-encapsulated glyco-NP suspensions were subjected to high-speed centrifugation of 10,000 rpm for 30 min. The supernatant containing the unencapsulated ampicillin was isolated. The ampicillin content in the supernatant was assayed by HPLC and the encapsulation efficiency (EE) was calculated. Full details are given in the Supporting Information file.

Turbidimetric Assay

Concanavalin A (Con A) was dissolved in HBS buffer (10 mM HEPES, 90 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂, pH = 7.4) (1 mg/mL), and the resulting solution was gently mixed. Turbidity measurements were performed by adding the Con A solution (400 µL) to a dry quartz cuvette (500 µL volume, 1 cm path length). A solution of the glyco-NP of interest in HBS buffer (prepared by nanoprecipitation method as described above) was then added (100 µL at 2 mg/mL). Upon addition, the solution was mixed vigorously using a micropipette before placement in a Varian Cary-100 UV–Vis spectrophotometer. Absorbance data were recorded at 420 nm for 10 min.

Bacteriological experiments

The bacteriological studies of the glyco-NPs (both Glc-NPs and Gal-NPs) and the ampicillin loaded glyco-NPs were investigated using an *Escherichia coli* K-12 wild-type strain (W3110 / ATCC27325, F⁻, λ⁻, *rpoS*(Am), *rph*-I, *Inv*(*rrnD*-*rrnE*)), *Staphylococcus aureus* (3R7089 strain

Oxford / ATCC9144) and *Staphylococcus epidermidis* (laboratory strain from clinical isolate) as representative Gram-negative (*E. coli*) and Gram-positive (*S. aureus* and *S. epidermidis*) species. MIC was defined as the lowest concentration which completely inhibited bacterial growth after incubation at 37°C for 16 h with agitation. Absorbance measurements at A_{650nm} were obtained using a Biotek Synergy H4 Plate Reader. Full details are given in the Supporting Information file.

RESULTS AND DISCUSSION

We designed amphiphilic glycopolymers with different compositions (hydrophilic to hydrophobic block ratio) that could assemble in aqueous solution, using the nanoprecipitation method,^{28, 29} into NPs with sugar moieties presented on the surface. Our synthetic approach was based on the reversible addition fragmentation chain transfer (RAFT) polymerization of an activated ester, pentafluorophenyl acrylate (PFPA), followed by chain extension using n-butyl acrylate (BA) for subsequent modification with aminoethyl glycosides (Fig. 1A). We first polymerized PFPA using benzyl 2-hydroxyethyl carbonotrithioate (BHECTT) as a chain transfer agent. The resulting pPFPA macroRAFT agents were then used to polymerize BA to generate block copolymers with different compositions as revealed by ¹H-NMR spectroscopy (Fig. S1, supporting information). After purification by reprecipitation, the block copolymers were analyzed by SEC which showed monomodal distributions with dispersities of about ca. 1.2. The RAFT end group on the block copolymers was removed by treatment with azobisisobutyronitrile (AIBN). The block copolymers were subsequently modified by reaction with 2'-aminoethyl-β-D-glucopyranoside or 2'-aminoethyl-β-D-galactoside. Under optimized experimental conditions, a high yield with total consumption of pentafluorophenyl ester as revealed by ¹H- and ¹⁹F-NMR and

FTIR spectroscopy were achieved (Fig. S2–4, supporting information). The final glycopolymers were characterized by SEC as shown in Table S1 (supporting information).

Glyco-NPs were prepared *via* the nanoprecipitation method and characterized by determining their diameter, external morphology and ampicillin encapsulation efficiency. The results showed that as the DP of the hydrophobic segment (pBA) increases, the diameter of the obtained NPs also increases (Table 1). Glucopolymer p(NβGlcEAM₁₅-*b*-BA₁₂₀) was found to form the largest NPs with a diameter of 272 ± 14 nm by DLS, Table 1. In addition, TEM was used to explore the morphology of the NPs. The obtained NPs are roughly spherical in shape; the apparent diameter of the NPs by TEM was found to be higher than their diameter in the hydrated state, most probably because of their flattened structure (Fig. 1B and 1F). Encapsulation of ampicillin led to a small increase in NP diameter (Table 1, entries 4 and 5). We therefore employed NPs made from glycopolymer p(NβGlcEAM₁₅-*b*-BA₁₂₀) in this study which showed the largest diameter and were expected to possess the highest drug loading capacity. The ampicillin encapsulation efficiency (EE) of these NPs was found to be 47 %.

Table 1. DLS measurements for the obtained glyco-NPs.

Sample	Size by DLS (nm) \pm SD	Polydispersity, PI \pm SD
p(NβGlcEAM ₁₅ - <i>b</i> -BA ₃₀)	145 ± 2	0.219 ± 0.022
p(NβGlcEAM ₁₅ - <i>b</i> -BA ₄₅)	197 ± 6	0.229 ± 0.018
p(NβGlcEAM ₁₅ - <i>b</i> -BA ₇₅)	218 ± 8	0.227 ± 0.027
p(NβGlcEAM ₁₅ - <i>b</i> -BA ₁₂₀)	272 ± 14	0.234 ± 0.032
Ampicillin loaded p(NβGlcEAM ₁₅ - <i>b</i> -BA ₁₂₀)	302 ± 23	0.297 ± 0.026

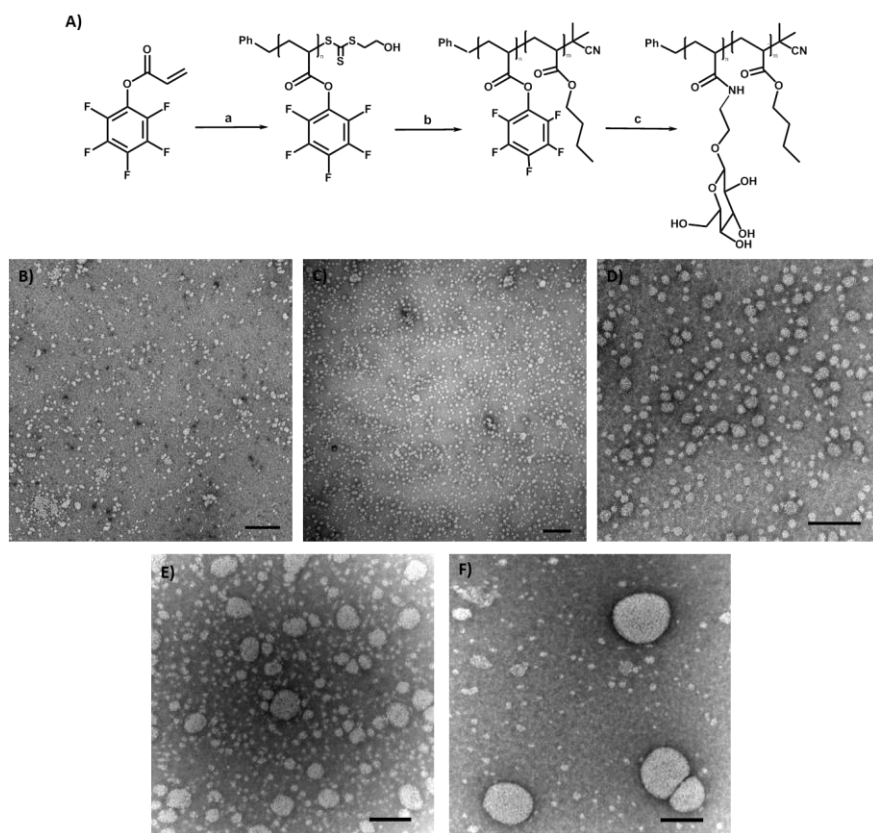


Figure 1. A) Schematic representation of amiphilic glycopolymers synthesis (Conditions: a) BHECTT and AIBN in benzene at 70 °C for 6 h, b) BA and AIBN in benzene at 70 °C for 6 h followed excess AIBN at 70 °C for 6 h, c) 2'-aminoethyl-β-D-glucopyranoside and TEA in DMF-water at 30 °C for 18 h. B)-F) TEM images of glyco-NPs made from B) p(NβGlcEAM₁₅-

b-BA₃₀), C) p(NβGlcEAM₁₅-b-BA₄₅), D) p(NβGlcEAM₁₅-b-BA₇₅), E) p(NβGlcEAM₁₅-b-BA₁₂₀), and F) ampicillin loaded glyco-NPs made from P(NβGlcEAM₁₅-b-BA₁₂₀). Scale bar = 200 nm.

The typical *in vitro* release profile of a drug from NPs often displays two distinct regions; an early, rapid release, while the remainder is liberated slowly during an extended period of time. The early release represents the loss of surface-associated and poorly entrapped drug. The magnitude of this burst effect is dependent on the quantity of drug bound to the outer surface of the NPs. However, the drug release from within the NP core is controlled by diffusion.³⁰ In our case, the percentage cumulative ampicillin release versus time plot did not show an initial burst release phase. Instead, slow and constant zero-order ampicillin release kinetics was observed. The cumulative ampicillin release from NPs after 21 days was found to be 56 % (Fig. 2).

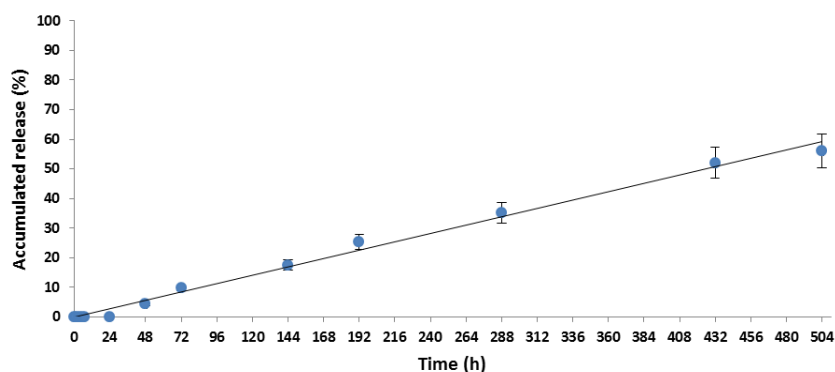


Figure 2. *In vitro* release profile of ampicillin-loaded P(NβGlcEAM₁₅-b-BA₁₂₀) glyco-NPs in PBS (pH 7.4) at 37 °C.

The ability of the glycosylated NPs to bind to the glucose/mannose specific lectin Concanavalin-A (Con-A) was evaluated using a well-established turbidimetric assay.³¹ In this assay, the Con-A tetramer is mixed with an excess of the multivalent ligand under investigation, inducing rapid precipitation. The change in turbidity is related directly to the formation of Con-A clusters in solution mediated by the appropriate multivalent ligand. The glucose-containing NPs were found to bind readily to Con-A; however, galactose-containing NPs did not show any binding affinity (Fig. 3). These results indicate that D-glucose moieties are presented on the surface of the NPs in a densely multivalent manner and are available for lectin binding. These results also show the potential of the glyco-NPs to bring about targeted delivery to cells displaying glucosyl-binding lectins.

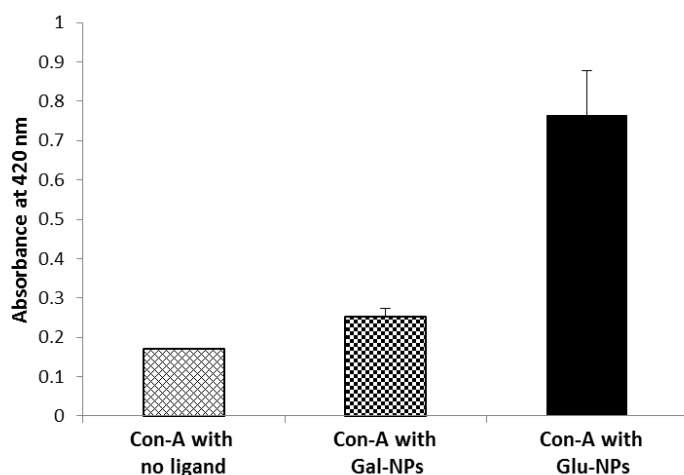


Figure 3. Assessment of the binding of NPs to Con-A by turbidimetry, from left to right: Con-A alone (control); Con-A plus glucosylated nanoparticles; Con-A plus galactosylated nanoparticles.

Commented [NC1]: Replace Glu with Glc

We performed bacterial minimal inhibitory concentration (MIC) assays³² using both glucosylated and galactosylated NPs (Glc-NPs and Gal-NPs, respectively), ampicillin loaded Glc-NPs and free ampicillin against representative Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*) bacteria. Unloaded Glc-NPs and Gal-NPs showed no significant antibacterial activity against these bacterial species (Fig. 4A-C), although there was some indication of a reduction in growth in the 5-25 µg/ml concentration range for Glc-NPs mixed with *E. coli* (Fig. 4A). Visual inspection of the plates revealed that this is due to aggregation of *E. coli* correlating with an increased Glc-NP concentration (Fig. 4D and also evident in the increased absorbance at the highest Gal-NP concentrations in Fig. 4A) (no aggregation was evident in any other wells). Carbohydrate recognition sites have been identified on the pili of *E. coli*. The FimH protein, present in the W3110 strain used here³³ which is exposed at the tip of the pili, has binding specificity for glucose and mannose on human cell surfaces¹⁷ and so this is a likely explanation for the *E. coli* aggregation observed with Glc-NPs but not with the non-binding Gal-NPs. The affinity of FimH for galactose is 10-fold lower than that for glucose in a surface plasmon resonance assay³⁴.

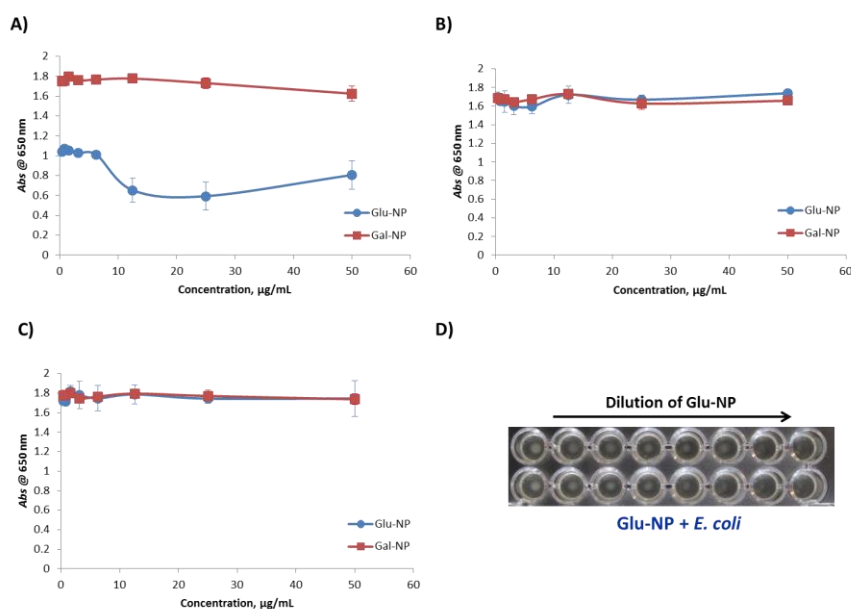


Figure 4. Evaluation of the growth and aggregation of bacteria in the presence of glucosylated nanoparticles (blue circles) and galactosylated nanoparticles (red squares): A) *E. coli*, B) *S. aureus*, C) *S. epidermidis*. D) Section of a 96-well plate showing bacterial aggregation of *E. coli* as a consequence of incubation with glucosylated nanoparticles for 16 h.

Commented [NC2]: Replace Glu with Glc

Representative examples of bacterial cultures grown in liquid media in the presence of Glc-NPs or ampicillin loaded Glc-NPs are shown in Fig. 5A (the results for *E. coli* only are shown, although similar results were obtained with *S. aureus* and *S. epidermidis*). Bacterial growth is evident in liquid media and in the presence of Glc-NPs (Fig. 5A, left and centre), while inclusion of ampicillin in the NPs completely inhibited the bacterial growth (Fig. 5A, right).

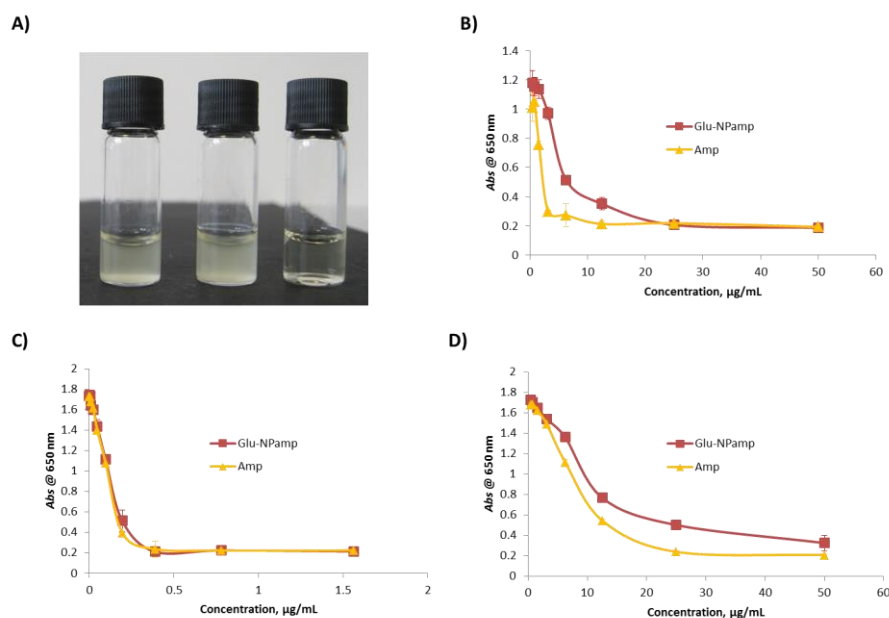


Figure 5. A) *E. coli* grown in liquid media (left), in the presence of Glc-NPs (centre) and ampicillin loaded Glc-NPs (right). Evaluation of bacterial growth in the presence of ampicillin loaded Glc-NPs (red squares) and ampicillin (yellow triangles). B) *E. coli*, C) *S. aureus*, D) *S. epidermidis*.

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Both ampicillin-loaded Glc-NPs and free ampicillin show antibacterial activity against *E. coli*, *S. aureus* and *S. epidermidis* (Fig. 5B-D). The MIC values of ampicillin loaded Glc-NPs and free ampicillin against different bacterial strains are shown in Table 2. Ampicillin-loaded Glc-NPs exhibited MICs 4-fold and 2-fold higher than free ampicillin against the Gram-negative strain *E. coli* and Gram-positive strains *S. epidermidis* and *S. aureus*, respectively. This presumably reflects the slower release of ampicillin from the Glc-NPs, compared to free ampicillin. The extent of bacterial killing is nonetheless quite remarkable given the small amount of ampicillin

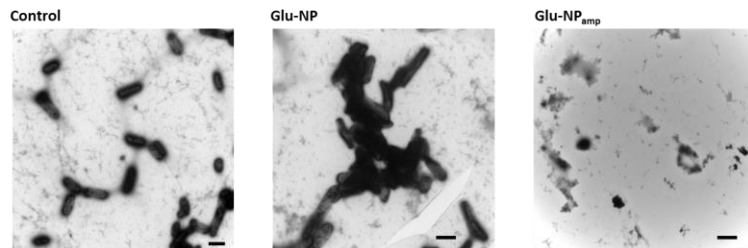
released over the 16h timeframe of the bacterial culture experiments (Fig. 2). The Glc-NPs alone display some antibacterial effect even without encapsulation of ampicillin, the reason for which requires further investigation.

Table 2. MIC assays on *E. coli*, *S. epidermidis* and *S. aureus*.

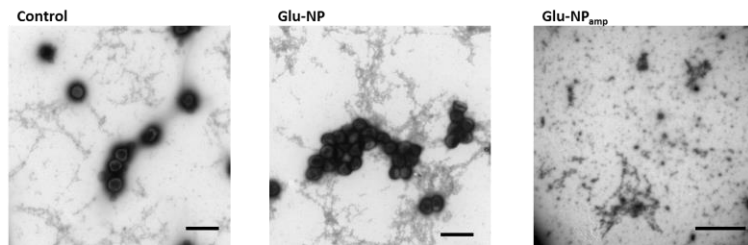
Strain	Glc-NP ($\mu\text{g mL}^{-1}$)	Glc-NP_{amp} ($\mu\text{g mL}^{-1}$)	Free Ampicillin ($\mu\text{g mL}^{-1}$)
<i>E. coli</i>	25	6.25	1.56
<i>S. epidermidis</i>	—	25	12.5
<i>S. aureus</i>	—	0.196	0.098

We also investigated bacterial morphology and aggregation in the presence or absence of Glc-NPs using TEM (Fig. 6A and 6B). The bacterial cultures were dried onto TEM grids, stained with uranyl acetate, and imaged using TEM. The resulting micrographs confirm that bacteria in the presence of Glc-NPs form significantly more aggregates compared to the control experiment without addition of Glc-NPs. Indeed, these TEM micrographs are representative of each sample as indicated by the collected statistical data (Fig. 6C and 6D).

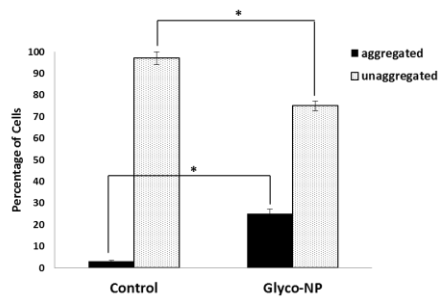
A) *E. coli*



B) *S. aureus*



C) *E. coli*



D) *S. aureus*

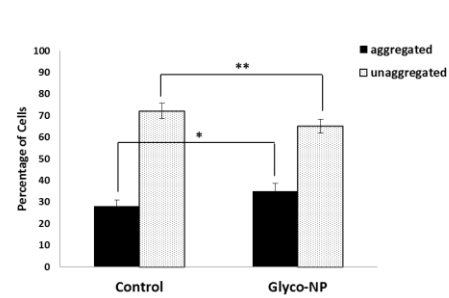


Figure 6. TEM images showing A) *E. coli* and B) *S. aureus* in the presence or absence of Glc-NPs. Cells were stained with uranyl acetate. Quantification of bacterial aggregation from TEM images for C) *E. coli* and D) *S. aureus* cells in the presence or absence of Glc-NPs. * denotes *p*

value < 0.15; ** p value < 0.0001 as determined by Student's t test (15 fields of view per experiment)

We refer to any cluster of ten or more cells attached to each other as “aggregated”; fewer than ten associated cells was defined as “unaggregated”. Based on this collected data, we noted a marked increase in the aggregation percent of *E. coli* cells in the presence of Glc-NPs compared to the control experiment without Glc-NPs (Fig. 6C). This was also the case for *S. aureus* cells (Fig. 6D), although the difference between the aggregation percentage of cells in the presence of Glc-NPs and in their absence (control) was much less obvious. The suggested explanation for *E. coli* aggregation is the presence of the Glc-binding FimH protein present on the tips of the bacterial pili. No such Glc-binding protein has been identified for *S. aureus*, so the significant ability of the Glc-NPs to aggregate these bacteria is as yet unexplained. These results are consistent with the MIC and culture assays presented earlier. We suggest that this bacterial aggregation induced by the glyconanobiotics accounts for their antibacterial effect, perhaps by facilitating release or delivery of the encapsulated antibiotic.

CONCLUSIONS

In this study, glycosylated polymeric nanoparticles (glyco-NPs) composed of a poly(n-butyl acrylate) (pBA) core and a poly(N-2-(β -D-Glucosyloxy)ethyl acrylamide) (pN β GlcEAM) corona were prepared by nanoprecipitation in PBS of preformed RAFT block copolymers. The antibiotic ampicillin was encapsulated in the core of these glycosylated NPs and released following a zero-order kinetic profile. The glycopolymer chains were presented to the outside environment of the NP and could bind specifically to the lectin Con A. The glycosylated NPs

were bioactive, exhibiting significant adhesive interactions with several bacterial strains (*E. coli*, *S. epidermidis* and *S. aureus*) as judged by the ability to induce bacterial aggregation. In the case of *E. coli*, glucosylated NPs were able to induce substantial bacterial aggregation compared to the galactosylated analogues. The ability of our glycosylated NPs to display the dual function of bacterial aggregation and antibiotic delivery is potentially a powerful route to produce antimicrobials with improved efficacy. Indeed, it was found that bacterial killing abilities only slightly lower than that of free ampicillin were obtained, despite the much lower instantaneous concentration of antibiotic in the vicinity of the glyco-NPs. By tuning the carbohydrate epitope on the NP tethers through polymer design and hence affinities in cell aggregation, a new class of *glyconanobiotics* may emerge.

ASSOCIATED CONTENT

Supporting Information. Materials and instrumentation used; detailed experimental procedures; characterization data for block glycopolymers; calculation of ampicillin encapsulation efficiency. This information is available free of charge on the ACS Publications website at DOI: xxxx.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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